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HEINZ SPECHT

DISSERTATION SUBMITTED TO THE BOARD OF UNIVERSITY STUDIES OF THE  
JOHNS HOPKINS UNIVERSITY IN CONFORMITY WITH THE  
REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

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## AEROBIC RESPIRATION IN SPIROSTOMUM AMBIGUUM AND THE PRODUCTION OF AMMONIA

HEINZ SPECHT

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THREE FIGURES

### INTRODUCTION

The ciliate, *Spirostomum ambiguum*, has been variously regarded as an aerobe and as a facultative or obligatory anaerobe, since Ehrenberg's first description of it. Observations on its natural habitat (a medium of rotting sapropelic slime rich in hydrogen sulfide) indicated to Lauterborn ('01) an anaerobic nature, but Pütter ('04) believed it an aerobe whose survival depends upon a narrow optimal range of oxygen tensions. Bishop ('23) attributed difficulties encountered in artificial culturing to a dependence upon fairly high tensions of hydrogen sulfide and low tensions of oxygen. She suggested that these two factors might act indirectly, possibly through the bacterial flora which forms its chief food. Saunders ('24) concluded that oxygen and hydrogen sulfide tensions have little to do with survival, and contended that Pütter's observations are to be explained in terms of the hydrogen-ion concentration of the medium, which the latter altered by aeration. He drew no conclusion on the question of anaerobiosis. (A treatise on "Die Kardinalkonzentrationen der lebensnotwendigen Stoffe," by Pütter ('27) contains references to his earlier work ('04) cited above, but apparently overlooks the criticisms and the work of Saunders.)

In view of the fact that no measurements of the rate of  $O_2$  consumption by *Spirostomum ambiguum* have been made, it is evident that the conclusions, themselves contradictory, based on the foregoing work, are not well founded.

#### MATERIAL AND METHODS

The spirostoma were obtained shortly before use either directly from pools or from cultures kept in the laboratory under regulated conditions.

The spirostoma in the pools (Woods Hole, Massachusetts) were in a quiescent condition. The solution in which they lived was clear with a hydrogen-ion concentration varying between pH 6.7 and 6.5. They were kept in pool water, either with or without the debris collected with them. Division was not observed, though the animals appeared to be in excellent condition. Some of them were used to inoculate laboratory cultures. The composition of the culture medium was that of the most successful of a series of trial solutions. This medium consisted of a hay-wheat infusion containing a table-spoonful of fresh cow manure per liter and an inoculum of bacteria from the Woods Hole stock (Specht, '34).

The animals were prepared for experimentation in the following manner: those obtained directly from the pools were first concentrated by means of a pipette or by a gentle centrifuging. Then they were put into a relatively large quantity of Knopf solution (no. 1—compare Collecting Net, 1930), stirred up and again concentrated. This process was repeated at least three times and resulted in the elimination of practically all free bacteria, as was demonstrated by tests of the  $O_2$  consumption of samples of the last wash water. Those animals obtained from the laboratory culture (pH 7.4) were washed in the same way, but in a phosphate buffer (Clark, '20), pH 7.4, diluted 1:100 with distilled water. In this buffer solution the spirostoma stay suspended for long periods, swimming and contracting in essentially the same manner as in the culture medium. It has been found since this work that Peter's medium (Hetherington, '33), pH 7.4—7.6, is better than the diluted phosphate buffer.

The apparatus used in the measurement of respiration consisted of a set of Warburg manometers with a shaking device and constant temperature bath described by Gerard ('31). Some of the experiments were made with a similar apparatus constructed by the author (Specht, '34). The temperature of the bath was set at 25°C. and maintained at  $\pm 0.01^\circ\text{C}$ . The speed of the shaking mechanism was set at 60 cycles per minute.

A series of measurements of  $\text{O}_2$  consumption and  $\text{CO}_2$  production in experiments in which the number of animals tested varied from 500 to 6000 in 2 cc. of fluid indicated that the rate of respiration is constant above about 1000 per cubic centimeter, and increases at an increasing rate below this value until a maximum is reached. In view of this fact, the number of animals per sample was chosen so that there were approximately 1000 per cubic centimeter. The procedure adopted in conducting the experiments is essentially the same as that of Warburg ('23, et seq.).

The microgasometric method used involves measuring the change in pressure of a gas in a constant volume. It is assumed that  $\text{O}_2$  is consumed and  $\text{CO}_2$  is given off during the course of the experiment. The presence of alkali in the inset of the respiration vessel keeps the  $\text{CO}_2$  tension at zero. A decrease in pressure in the vessel is converted into an equivalent volume assumed to be that of the  $\text{O}_2$  consumed. The comparison of pressure values obtained in the manner indicated above and those obtained similarly from a vessel in which no alkali is present gives, presumably, the increase in pressure due to the liberation of  $\text{CO}_2$ , and these values are in turn converted to the equivalent volumes assumed to be  $\text{CO}_2$  produced. This method, in addition to being a micro-method, is subject to errors resulting from the assumptions made above.

#### AMMONIA PRODUCTION

During preliminary experiments it was noted that a positive pressure developed when it was expected that a negative

pressure, due to  $O_2$  consumption, should appear; and that conversely the positive pressure of  $CO_2$  was extremely low under similar circumstances. It was also evident that the deviation from the average respiratory rate was rather large, especially in regard to  $CO_2$  production. It seemed possible that this deviation resulted from the interference of some volatile substance liberated during the course of the experiment. Another series of experiments was made in which control of this factor was attempted through the use of 0.1 N HCl as absorbent (0.1 N  $H_2SO_4$  was later used in several experiments

TABLE 1  
*Respiration and R.Q. in Spirostomum ambiguum under various conditions*

|                                      | ABSORB-<br>ENT  | MM. <sup>3</sup> /HR./1000<br>IN AIR |              | MM. <sup>3</sup> /HR./1000<br>IN O <sub>2</sub> |      | MM. <sup>3</sup> /HR./1000<br>IN N <sub>2</sub> 0.5 PER<br>CENT O <sub>2</sub> |              | IN N <sub>2</sub> (DIS-<br>INTEGRATED) |      |
|--------------------------------------|-----------------|--------------------------------------|--------------|-------------------------------------------------|------|--------------------------------------------------------------------------------|--------------|----------------------------------------|------|
|                                      |                 |                                      | S.D.         |                                                 | S.D. |                                                                                | S.D.         |                                        | S.D. |
| O <sub>2</sub>                       | HCl             | 2.59<br>2.10                         | 0.51<br>1.39 | 3.92                                            | 1.02 | 1.86<br>0.76                                                                   | 1.55<br>1.42 | 0.07                                   | 0.44 |
| CO <sub>2</sub>                      | HCl             | 2.17<br>0.86                         | 0.87<br>1.84 | 3.80                                            | 1.43 | 2.53<br>1.53                                                                   | 2.40<br>3.51 | 2.79                                   | 2.04 |
| R.Q.                                 | HCl             | 0.84<br>0.24                         |              | 0.98                                            |      | 1.36<br>120                                                                    |              | Glycolysis                             |      |
| Per cent of<br>respiration<br>in air | O <sub>2</sub>  | 100                                  |              | 151                                             |      | 71                                                                             |              | 3                                      |      |
|                                      | CO <sub>2</sub> | 100                                  |              | 175                                             |      | 70.5                                                                           |              | 129                                    |      |

and gave identical results). The acid was placed in a stoppered sidearm opening into a main chamber of the respiration vessel (Warburg, '23; Gerard, '31).

The results, as presented in table 1 and figure 1, demonstrate that a substance absorbable by HCl is liberated into the vapor above the metabolizing organisms. The effect of absorbing this substance is not only to increase the apparent  $O_2$  consumption, but also to reduce considerably the variation between the results obtained in a series of experiments, as indicated by the standard deviation.

Richardson ('29) has pointed out that excretory nitrogen is usually not considered in the measurements of the respira-

tory quotient of single cells or small groups of cells, and since it affects not only the theoretical respiratory quotient, but possibly the practical determination, it is an important consideration. Tashiro ('22) found that the indicator method for ascertaining  $\text{CO}_2$  production of nerves is subject to a considerable error due to the production of volatile  $\text{NH}_3$ , and Lee and Tashiro ('22) demonstrated the same for muscle.

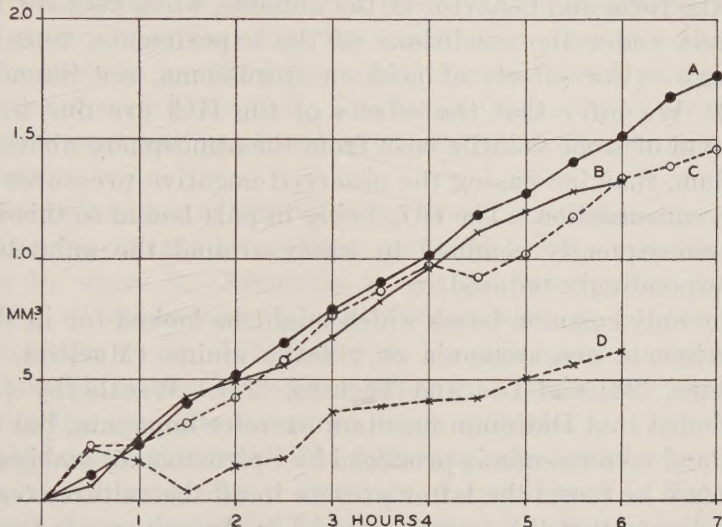


Fig. 1 Respiration in *Spirostomum ambiguum* in air at 25°C. C, average of five experiments for  $\text{O}_2$  consumption without HCl; A, average of five experiments for  $\text{O}_2$  consumption with HCl; D, average of four experiment for  $\text{CO}_2$  production without HCl; B, average of four experiments for  $\text{CO}_2$  production with HCl. (Note that the results indicate that the respiratory rates are higher with than without HCl in the respiration vessels. It is demonstrated in the text that this is not due to the animals, but to the method.)

Recently, and subsequent to the observations on *Spirostomum* presented here, a paper by Gerard and Hartline ('34) presented a review of literature on the production of ammonia in nerve tissue. The substance present in cultures of *Spirostomum* manifests itself in the absence of HCl as a positive pressure decreasing the negative pressure due to  $\text{O}_2$  consumption, or as a negative pressure decreasing the positive pressure due to  $\text{CO}_2$  production. In the presence of HCl this

substance is apparently absorbed, increasing the values obtained for  $O_2$  consumption and  $CO_2$  production. That these results are due to the direct effects of the HCl on the animals or the medium is highly improbable, since the acid is separated from the fluid medium and is in contact only with gaseous medium above it. The distillation of HCl from 0.1 N solutions is negligible at  $25^\circ C.$ , and the form and behavior of the animals, when kept for long periods under the conditions of the experiments, remained normal. (For effects of acid on spirostoma, see Saunders, '24.) We infer that the effects of the HCl are due to the removal of some volatile base from the atmosphere above the medium, thus increasing the observed negative pressures due to  $O_2$  consumption. The  $CO_2$ , being in part bound to this base and consequently confined to water around the animals, is correspondingly reduced.

The only common bases which might be looked for in these experiments are ammonia or volatile amine (Macleod, '30; Tashiro, '22; and Lee and Tashiro, '22). Weatherby ('29) concluded that *Didinium nasutum* excretes ammonia, but that urea and no ammonia is produced by *Spirostomum ambiguum*, although he found the latter present in all the cultures tested. He believed that the ammonia found in the cultures is formed through the activity of bacteria on urea excreted by the spirostoma.

Tests made in these experiments with Nessler's reagent indicated that, in accord with Weatherby's contention, there was an appreciable amount of ammonia in both the natural media and the cultures used. But these tests do not show whether the animals produced the ammonia or not. Accordingly a thick suspension of spirostoma was repeatedly washed in Knopf's solution as described above, until no ammonia could be detected in the wash water. The animals, together with 250 cc. of this last wash, were then put into one flask and equal quantities of the wash water into two other flasks. To one of the latter was added a relatively large amount of urea. The urea solution was tested for ammonia

with a negative result. The three flasks were left for 6 hours at room temperature ( $22^{\circ}$  to  $24^{\circ}\text{C.}$ ), when samples were drawn from each and tested for ammonia. The samples from the flask containing spirostoma gave a decidedly positive test, while those from the other two flasks gave negative tests. Bacterial contamination was practically equal in all the flasks, since the wash water from the animals was used as solvent. The result indicates either that the Spirostoma actually produce ammonia or that they have some very immediate effect on the bacteria enabling the latter to break down urea when present in small quantities. The former seems to be the more probable explanation, since there were relatively few bacteria present.

The explanation of why Weatherby did not obtain ammonia in his experiments is perhaps revealed by the experiments given in figure 3. Ammonia is not produced when a suspension of spirostoma is equilibrated with  $\text{O}_2$  at tensions greater than in air. Weatherby, in his experiments, repeatedly washed his animals on filter paper and thoroughly exposed them to the air. His failure to detect ammonia was therefore possibly due to this fact. The following experiment was made to answer this question. Two lots of spirostoma in Knopf's solution were aerated for 14 hours, one with air and one with  $\text{O}_2$ . Then the  $\text{O}_2$  consumption and  $\text{CO}_2$  production were measured, with and without  $\text{HCl}$ . No appreciable difference was detected between the values obtained from the air-washed and oxygen-washed cultures, nor from the values obtained in pure oxygen as cited below. It appears, therefore, that the excretion of ammonia is augmented by lack of oxygen and minimized by an abundance of it.

#### RESPIRATION AT NORMAL $\text{O}_2$ TENSION

The averaged values for the  $\text{O}_2$  consumption and  $\text{CO}_2$  production from five experiments with  $\text{HCl}$ , using media equilibrated with air, are presented in figure 1, curves A and B, respectively. (It sometimes happens that disintegration of the whole sample in the respirometer takes place, accompanied

by a marked increase in the rate of  $O_2$  consumption and  $CO_2$  production. The cause of this phenomenon is undetermined. The respiratory rate attained under such circumstances compares well with those obtained in high tensions of oxygen, as will be seen later. Experiments terminating in this manner were, of course, excluded from those the values of which were used for the calculation of the normal rate of respiration.) To contrast with the values given above, average values obtained in four experiments without HCl are also given in figure 1, curves C and D. Data on the average rate of respiration are given in table 1 under 'air.' The difference between the average rates recorded in table 1 for the  $O_2$  consumption with and without HCl is 0.49 cu.mm., and for the  $CO_2$  production 1.31 cu.mm. Even more striking is the decrease in the standard deviation of these values: from  $\pm 1.39$  to  $\pm 0.51$  for the former and from  $\pm 1.84$  to  $\pm 0.87$  for the latter.

#### RESPIRATION AT LOW $O_2$ TENSION

The following experiments were made as described above, with the exception that commercial tank  $N_2$  containing 0.5 per cent  $O_2$  was passed through all the manometers and their attached vessels. By flushing the vessels for a period of 5 minutes and agitating the contained liquids, the concentration of  $O_2$  in the gas in the manometers was reduced to approximately 1 part in 200 by volume. The concentration was, however, not constant from experiment to experiment, as is borne out by duration of survival of the animals, though it is probable that in the same experiment different manometers contained nearly the same concentration of  $O_2$ . But it was necessary to find some observable point in time, as the  $O_2$  tension diminished, at which the condition of the manometers and the animals in each was the same. Shortly after the calculated amount of  $O_2$  initially present in the vessels was gone, a total disintegration of the animals seemed to follow—an invariable result of testing at low oxygen tensions for a protracted period. This end point was used to correlate the results from the different experiments.

The average values for  $O_2$  consumption and  $CO_2$  production obtained in four experiments, in which HCl was present in the respiration vessels, are given in figure 2, curves A and B, respectively. The values for the  $O_2$  consumption and the  $CO_2$  production obtained without HCl in the respiration vessels are

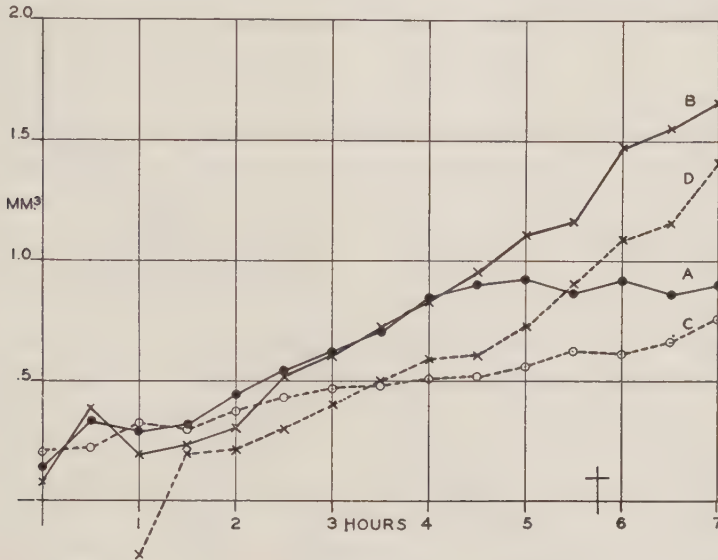


Fig. 2 Respiration in *Spirostomum ambiguum* in 0.5 per cent  $O_2$  + 99.5 per cent  $N_2$  at  $25^\circ C$ . C, average of three experiments for  $O_2$  consumption without HCl; A, average of four experiments for  $O_2$  consumption with HCl; D, values of a single experiment for  $CO_2$  production without HCl; B, average of four experiments for  $CO_2$  production with HCl. (+), point at which complete disintegration of sample of animals has occurred. (Note that the slope of the curve, A, falls off nearly an hour before this and that no increase in respiration is effected by the dissolution of the animals, indicating that all available  $O_2$  in the vessel has been removed.)

given, respectively, in curves C (three experiments) and D (one experiment). The point of the abscissa in figure 2, which is marked with a cross, is the time at which total disintegration of the animals occurred. The average rates of  $O_2$  consumption and  $CO_2$  production for this period are given in table 1 under ' $N_2$  + 0.5 per cent  $O_2$ ,' using only the observations up to the time that disintegration began.

Complete exhaustion of  $O_2$  is indicated by the fact that when the animals disintegrate there is no jump in the rate of  $O_2$  consumption, such as occurs when disintegration takes place at normal tensions of  $O_2$ . This fact is further shown by the following experiments: air was introduced after 2 hours

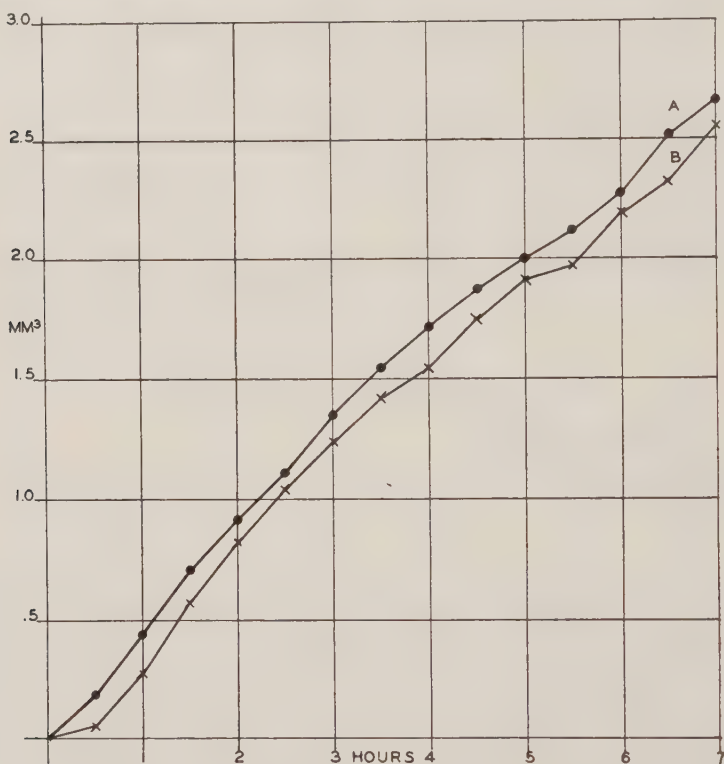


Fig. 3 Respiration in *Spirostomum ambiguum* in pure  $O_2$  at  $25^\circ C$ . A, average of two experiments for  $O_2$  consumption with HCl; B, for  $CO_2$  production. (The values obtained without HCl do not differ from these and are not plotted.)

into a manometer which had been flushed with  $N_2$  as described above, and in which a few animals had disintegrated and the rate of  $O_2$  consumption was falling off slightly. As a result of the introduction of  $O_2$ , the rate of consumption immediately rose, approximating that in pure  $O_2$ , and complete disintegration did not occur until some 6 hours later.

In these experiments, as in those of the preceding section, it is evident that HCl brings about a marked increase in the values obtained for respiratory measurements (table 1).

#### RESPIRATION AT HIGH O<sub>2</sub> TENSION

In these experiments the concentration of O<sub>2</sub> was raised to nearly 100 per cent by passing through the manometer O<sub>2</sub> prepared for dental work.

The average values for O<sub>2</sub> consumption and CO<sub>2</sub> production obtained in two experiments with HCl in the vessels are presented in figure 3, curves A and B respectively. The values obtained with and without HCl are not perceptibly different under this condition. The average rate of respiration is given in table 1 under 'O<sub>2</sub>.'

From figure 3 it will be seen that the high rate of respiration is induced very early after the beginning of the experiment and that it proceeds at a fairly constant rate for the duration of the experiment. In this O<sub>2</sub> enriched atmosphere the spirostoma were tested for 8 or 10 hours, but they became abnormal in shape and sometimes disintegrated. The rate of O<sub>2</sub> consumption and CO<sub>2</sub> production is not increased by the dissolution of the animals.

#### DISCUSSION

Data on the O<sub>2</sub> consumption of various unicellular forms collected from several observers are given in table 2. The values obtained for *Spirostomum ambiguum* are low in comparison with those obtained for the comparable ciliate, *Paramecium*, but higher than those obtained for the non-ciliate forms, when compared on the basis of respiration per unit volume. Comparisons on unit weight, unit surface, or unit volume are all misleading in that they do not compare the essential respiring substance of the cell. The highly vacuolar nature of the protoplasm in *Spirostomum* leads one to doubt the value of comparisons based on overall dimensions and weight. It is, however, of more value to consider these values in the light of the natural conditions under which the various

forms occur. As mentioned in the introduction, *Spirostomum ambiguum* seems to be able to live in media notoriously poor in  $O_2$ , forming dense clusters in the debris of the bottom of its habitat—yet it disintegrates in the complete absence of  $O_2$ .

TABLE 2  
*Comparison of respiratory rates of several protozoa with those of  
Spirostomum ambiguum*

| ORGANISM AND<br>SOURCE                                                   | MM. <sup>3</sup> O <sub>2</sub> /HR.<br>PER<br>INDIVIDUAL | MM. <sup>3</sup> CO <sub>2</sub> /HR.<br>PER<br>INDIVIDUAL | MM. <sup>3</sup> O <sub>2</sub> /HR.<br>PER VOL. OF<br>SPIROSTOMUM | MM. <sup>3</sup> CO <sub>2</sub> /HR.<br>PER VOL. OF<br>SPIROSTOMUM |
|--------------------------------------------------------------------------|-----------------------------------------------------------|------------------------------------------------------------|--------------------------------------------------------------------|---------------------------------------------------------------------|
| <i>Spirostomum ambiguum</i> ,<br>(Specht, this paper)                    | 0.0025                                                    | 0.0013                                                     | 0.0025                                                             | 0.0013                                                              |
| <i>Paramecium caudatum</i> ,<br>(Howland, et al., '31)                   | 0.00033<br>0.00049                                        |                                                            | 0.00990<br>0.01490                                                 |                                                                     |
| <i>P. caudatum</i> , (Kalmus,<br>'27-'28)                                | 0.00520                                                   |                                                            | 0.15600                                                            |                                                                     |
| <i>P. caudatum</i> , (Necheles,<br>'24)                                  | 0.00385                                                   |                                                            | 0.11550                                                            |                                                                     |
| <i>P. caudatum</i><br>Starved                                            | 0.00004                                                   |                                                            | 0.00120                                                            |                                                                     |
| Fed<br>(Lund, '18-19)                                                    | 0.00014                                                   |                                                            | 0.00420                                                            |                                                                     |
| <i>P. caudatum</i> during cycle of<br>conjugation (Zweibaum,<br>'21-'22) | 0.00074<br>0.00348<br>0.00068<br>0.00225                  |                                                            | 0.002200<br>0.10440<br>0.00400<br>0.06750                          |                                                                     |
| <i>P. aurelia</i><br>Barratt, '05)                                       | (0.00015)<br>assumed                                      | 0.00012                                                    | (0.00900)                                                          | 0.00720                                                             |
| <i>Amoeba proteus</i><br>(Emerson, '29)                                  | 0.147/mm. <sup>3</sup>                                    |                                                            | 0.00175                                                            |                                                                     |
| <i>Aetinosphaerium</i> (Howland<br>et al., '31)                          | 0.00113                                                   |                                                            | 0.00028                                                            |                                                                     |

*Paramecium*, on the other hand, gathers at the surface of the media it inhabits, where the  $O_2$  concentration is at a maximum. It may be inferred that the tolerance to low  $O_2$  tensions exhibited by *Spirostomum ambiguum* is linked with the fact that its normal  $O_2$  consumption is not very great in comparison with that of other ciliates.

Some interest attaches itself to the R.Q. obtained in respiratory measurements on protozoa. In general, they have been found to be of such a low order that they must be regarded with suspicion. For this reason  $O_2$  consumption alone has been generally determined and  $CO_2$  calculated from an assumed R.Q., usually 0.8, taken over from metazoan work. That such an assumption is justified is shown by the fact that the R.Q. determined for *Spirostomum ambiguum* without HCl is 0.24 and with HCl 0.84. That is, the low R.Q. ordinarily obtained is apparently due to binding of  $CO_2$  in the liquid medium by  $NH_3$ . It is quite possible that the R.Q. obtained with HCl may still be too low, since in an atmosphere of  $O_2$  there is no appreciable  $NH_3$  formed and the R.Q. is 0.98. The equilibrium between  $CO_2$  and  $NH_3$  is probably such, even with HCl in the chamber, that the manometric method does not give the proper value for  $CO_2$  produced, neglecting that which is retained in solution in combination with  $NH_3$ .

#### SUMMARY

Measurement of the respiration in *Spirostomum ambiguum* by the usual manometric method with and without an acid (HCl) in the respiration vessels reveals a marked difference in both  $O_2$  consumption and  $CO_2$  production, due presumably to the presence of  $NH_3$  produced by the animals.

Values for the  $O_2$  consumption in air, pure  $O_2$ , and  $N_2 + 0.5$  per cent  $O_2$  are given and compared with data from the literature. It is pointed out that the presence of ammonia probably accounts for the low R.Q.s obtained in observations on this and other protozoa, and that the true R.Q. lies in the same range as that of metazoa.

The writer is indebted to Dr. R. W. Gerard and to the staff of the physiology course of the Marine Biological Laboratory of Woods Hole, Massachusetts, for their interest in this work and for many favors in the matter of apparatus and space, and to Prof. S. O. Mast for aid in preparation of the text.

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## VITA

Heinz Specht was born in Utica, N. Y., on March 9, 1907. He attended the public schools in Scotia and Schenectady, N. Y., and was graduated from Central High School in Schenectady, N. Y., in 1926. The next 4 years were spent at Princeton University, where in June, 1930, he received the degree of Bachelor of Science in Biology. During his first two summer vacations while at Princeton he was employed as research assistant in the Research Laboratories of the General Electric Company at Schenectady, N. Y., under Dr. S. Dushman. The following two summer vacations he was research assistant to Prof. Ulric Dahlgren at the Weir Mitchell Station of the Mount Desert Island Biological Laboratory in Maine. In the fall of 1930 he entered the School of Higher Studies at the Johns Hopkins University. He was assistant in General Biology part of this year and the following year, and in 1932-1933 held a University Scholarship. He spent the summer of 1931 in Maine as the collector for the Mount Desert Island Biological Laboratory and the following summer (1932) in Woods Hole, Mass.





